

Short communication

Systemic adjuvant effect of cholera toxin in the chicken

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Abstract

Cholera toxin (CT) is a well-known mucosal adjuvant in mammals, but it does not give conclusive results in chickens. Cells from the chicken immune system may be insensitive to CT activity. Our results showed that intravenously administered CT had strong immunomodulatory effects on chicken antigen-specific T- and B-cell immune responses. Seven and eight days post-inoculation (p.i.), chickens immunized with KLH and CT exhibited a faster and higher specific proliferative response in the spleen after *in vitro* restimulation than chickens immunized with KLH alone. At the same time, the specific antibody response in serum was significantly higher, with a strong IgG enhancement and a peak of IgA in chickens immunized with KLH and CT. The anti-KLH splenic antibody response *in vitro* involved a significant increase in specific IgG and IgA isotypes when CT was used as adjuvant. In conclusion, as in mammals, systemic CT demonstrated strong adjuvant properties in chickens enhancing T-cell priming *in vivo* and, thus, leading to increased specific antibody production, including IgA. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cholera toxin (CT) is an 87 kDa bacterial enterotoxin produced by *vibrio cholerae* and is composed of one A chain (CT-A: 27 kDa) and five B chains (CT-B: 12 kDa). CT exhibits strong mucosal adjuvant properties in mammals, greatly enhancing mucosal IgA and systemic IgG response as well as preventing oral tolerance of unrelated soluble antigens administered with CT perorally (Snider, 1995). The use of CT as an oral adjuvant in chickens has not to-date been very conclusive (Meinersmann and Porter,

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1993; Hoshi et al., 1995). Results have recently been obtained using antigen conjugated with CT and administered locally in the caecum (Vervelde et al., 1998). The poor adjuvant property of CT when administered orally in chickens might be related to a difficulty in passing through the intestinal barrier, either because of excessive degradation in the intestinal tract or an absence of receptor GM1 on the mucosa. In fact, binding of CT has been shown on the chicken intestinal mucosa after *in vivo* administration (Vervelde et al., 1998) and to be mediated via GM1 on isolated chicken intestinal epithelial cells (Hyun and Kimmich, 1984). Nevertheless, cells able to take up CT given orally and to transport it throughout the intestine have not yet been identified in chickens.

It is, however, possible that the cells involved in immune responses are not as sensitive to CT in chickens as in mammals. The aim of the present study was, therefore, to analyse the adjuvant capacity of CT administered systemically, thus by-passing the intestinal barrier, in terms of *in vivo* enhancement of antibody response and preferential IgA antibody switching.

2. Materials and methods

2.1. Chickens

All studies were performed on two-month-old White Leghorn chickens, homozygous for the B13 major histocompatibility haplotype, born and raised at INRA (PAP, 37 380 Nouzilly) under SPF conditions until experimental use.

2.2. Immunizations and experimental design

Cholera toxin and keyhole limpet hemocyanin (KLH) were purchased from Sigma. In experiment one, 16 chickens were inoculated intravenously with KLH (200 µg per chicken) mixed with CT (4 µg per chicken) and the other 16 with KLH alone. Spleen-specific proliferative response was tested on six chickens per group on days 7 and 8 post-inoculation (p.i.). Spleen-specific antibody production was tested on four chickens per group on Day 7 p.i. and on eight chickens per group on Day 8 p.i. Sera were taken on eight chickens per group on days 7 and 8 p.i. In experiment two, 28 chickens were immunized with KLH, half with CT and the other half without. Sera were taken on Day 7 (four chickens per group) and Day 10 p.i. (10 chickens per group).

2.3. Sera and spleen cells

Blood samples were obtained from chickens by cardiac puncture; allowed to clot for 4 h and centrifuged for 10 min at $3000 \times g$. The aliquots were kept at -20°C . Spleens were removed aseptically. Single-cell suspensions in 1.1X PBS pH 7.4 (Gibco) were prepared by gently teasing the organ on a steel sieve. Nucleated erythrocytes were eliminated by centrifugation at $400 \times g$. The leukocytes were used *in vitro* for proliferation and antibody production assays.

2.4. *In vitro* lymphocyte proliferation assay

Spleen cells were distributed in round bottomed 96-well microtiter plates (Falcon, Becton Dickinson) at 5×10^5 cells per well (200 μ l) in RPMI 1640 medium (Gibco) supplemented with L-glutamine (300 mg/ml, Gibco), penicillin (100 IU/ml, Gibco), streptomycin (100 μ g/ml, Gibco), bovine insulin (5 μ g/ml, Gibco), 2- β mercaptoethanol (5×10^{-5} M, Sigma) and 2.5% chickens serum. Soluble KLH was added at 25 μ g/ml and background controls were prepared with medium alone (six replicates for each). Cultures were kept for four days at 41°C in a humidified 5% CO₂ atmosphere. Tritiated thymidine (0.5 μ Ci, ICN, CA, USA) was added to each well for the final eight hours of culture and the plates were then frozen. Cells were harvested onto fiberglass filters after thawing. The radioactivity was measured in a β -counter (Kontron Betamatic V) and expressed as counts per minute (CPM).

2.5. *In vitro* antibody production

Spleen cells (5×10^6 /ml) were cultured in complete RPMI medium with 2.5% chicken serum, with or without KLH (0.1 μ g/ml), in 4.5 ml cell-culture tubes (Corning) for seven days at 40°C in a humidified 5% CO₂ atmosphere (3–4 replicates). Supernatants were obtained after centrifugation at $3000 \times g$ and kept at –20°C until use.

2.6. ELISA

Microtiter plates were coated with 10 μ g/ml soluble KLH in carbonate buffer (pH 9.6) (100 μ l/well) by incubation overnight at 37°C and then saturated with 2% bovine serum albumin (Sigma) (200 μ l/well) for 1 h. The wells were washed with PBS-Tween 20 (0.05%, Sigma). Samples (sera or culture supernatants: 100 μ l/well) diluted in PBS-Tween 20 (0.05%) (pH 7.2) were incubated for one hour at 37°C. After three washings with PBS-Tween 20, 100 μ l of horseradish peroxidase (HRPO) conjugated goat anti-chicken IgM μ -chain (1 : 2000; Bethyl Laboratories, Montgomery, TX) or HRPO-conjugated goat anti-chicken IgA α -chain (1 : 2000; Bethyl Laboratories) or alkaline phosphatase-conjugated rabbit anti-chicken IgG Fc fragment (1 : 10000; Jackson IR Laboratories, PA) was added for one hour at 37°C. Finally, color was developed using 100 μ l of 0.1 μ g ml⁻¹ solution of 2-2'-azino-bis (3-ethylbenzthiazoline 6- sulfonic acid) diammonium (Sigma) in citrate buffer (pH 4) for IgA and IgM determination, or 100 μ l of 1 mg ml⁻¹ solution of *p*-nitrophenylphosphate (Amresco, OH) in diethanolamine (pH 9.8) for IgG determination.

3. Results

Chickens immunized with KLH and CT demonstrated a high proliferative response to KLH *in vitro* by Day 7 p.i. (CPM 7124 ± 1500) whereas chickens immunized with KLH alone did not (826 CPM ± 486) (Fig. 1). The proliferative response of the first group increased dramatically by Day 8 p.i. (CPM 32245 ± 6951) and the second group began to develop a specific immune response (CPM 6534 ± 2833).

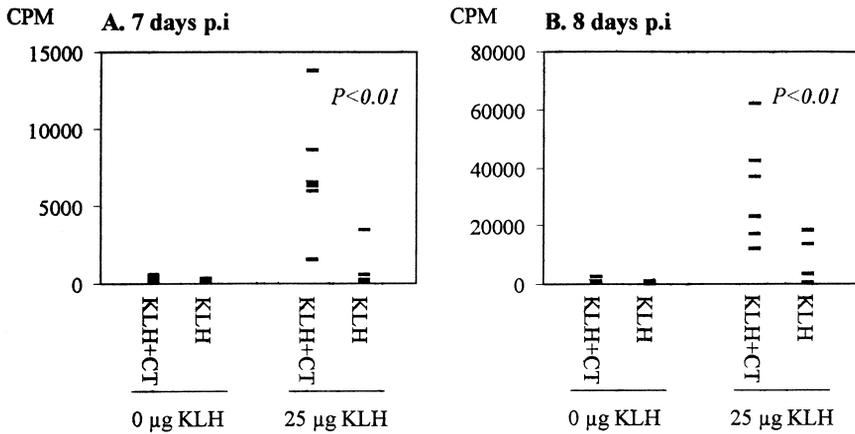


Fig. 1. CT given i.v. promotes antigen-specific proliferation of spleen cells in chickens immunized i.v. with KLH. Spleen cells were obtained on days 7 (A) and 8 (B) after i.v. immunization with KLH alone (200 µg) or KLH and CT (4 µg). They were cultured in medium alone (0 µg) or in the presence of KLH (25 µg/ml) for four days. Each point represents 3HdT uptake for one chicken (mean CPM of six replicates). (KLH + TC) values are compared to (KLH) values (six chickens per group).

In contrast to the specific spleen proliferative response, no difference was noted in specific antibody production in serum between Day 7 and Day 8 p.i. (experiment one) (Fig. 2). Co-injection of CT with KLH significantly enhanced the level of anti-KLH IgM ($p < 0.01$), IgG and IgA ($p < 0.001$) compared with immunization with KLH alone one week p.i. In experiment two, a different batch of KLH was used and the level of the immune response was lower than in experiment one. Nevertheless, chickens receiving KLH plus CT also exhibited significantly higher anti-KLH IgG and IgA responses at seven days p.i. than chickens immunized with KLH alone. At 10 days p.i., the IgG response reached the same level for both the groups, but the peak of anti-KLH IgA decreased in chickens immunized with KLH plus CT.

In the *in vitro* anti-KLH response (Fig. 3), spleen cells from KLH-immunized chickens did not produce any significant specific antibody production after restimulation with KLH (0.1 µg/ml) on Day 7 p.i. By that time, spleen cells from the chickens immunized with KLH plus CT were already producing significant anti-KLH IgM. Anti-KLH IgG and IgA were also produced, but as much in cultures stimulated with the antigen as in the non-stimulated ones, thus pin-pointing the existence *in vivo* at that time of cells in an activated state spontaneously secreting antibodies *in vitro*. By eight days p.i., spleen cells from chickens immunized with KLH and CT no longer spontaneously produced anti-KLH IgG and IgA in supernatants, and the level of re-stimulated specific IgG and IgA increased dramatically compared with chickens immunized with KLH alone (15- and 4.5-fold respectively).

4. Discussion

Our results in chickens demonstrated that CT injected intravenously so as to by-pass the intestinal barrier was able to exert very good adjuvant activity when co-injected with a

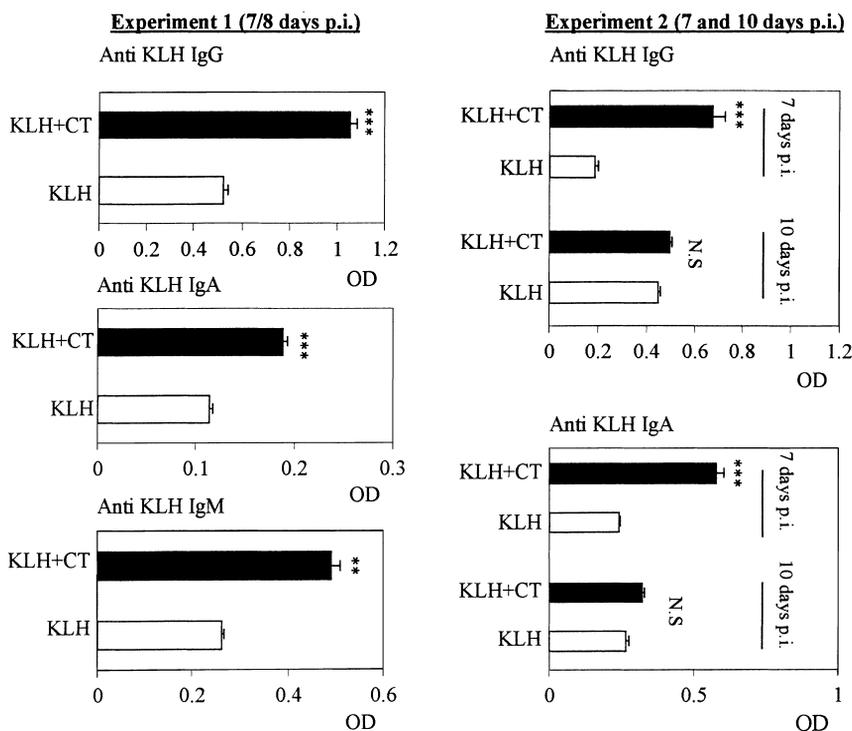


Fig. 2. Adjuvant effect of CT on anti-KLH IgG, IgA and IgM production in serum 7/8 days p.i. (Experiment 1) or 7 and 10 days p.i. (Experiment 2). Specific antibodies were detected by ELISA (1 : 10240 Exp. 1; 1 : 200 Exp. 2). (KLH + TC) values are compared to (KLH) values and statistical significance is indicated (N.S., nonsignificant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Results are expressed as mean O.D value (SEM) of eight chickens per group (Exp. 1) or four chickens per group on Day 7 p.i. and 10 chickens per group on Day 10 p.i. (Exp. 2).

soluble protein, such as KLH. The main effect was an earlier and higher antigen specific T-cell proliferative response in the spleen from Day 7 p.i., as was observed in mice with the same immunization protocol (Hornquist and Lycke, 1993). Thus, CT appears able to strongly promote antigen-specific priming of chicken CD4⁺ T lymphocytes in vivo. This was accompanied by more specific antibody production of all isotypes in the serum, including IgA. At the same time, spleen cells exhibited a higher capacity to produce specific anti-KLH IgG and IgA after antigen restimulation in vitro. A similar increase in specific antibody production in spleen is also observed in mice after simultaneous intravenous injection of CT and antigen (Vajdy et al., 1995).

Many immunomodulating and enhancing effects of CT have been reported on T-cell functions (Xu-Amano et al., 1994), B-cell differentiation (Lycke, 1993) and antigen presentation (Bromander et al., 1991, 1993) in mammals. However the mechanisms involved for the adjuvant action in vivo remain a matter of debate. Using CD4-deficient mice, Hornquist et al. (1995) demonstrated the absolute requirement of CD4⁺ T-cells to obtain the adjuvant effect of CT in vivo, whether given orally or intravenously. But, no

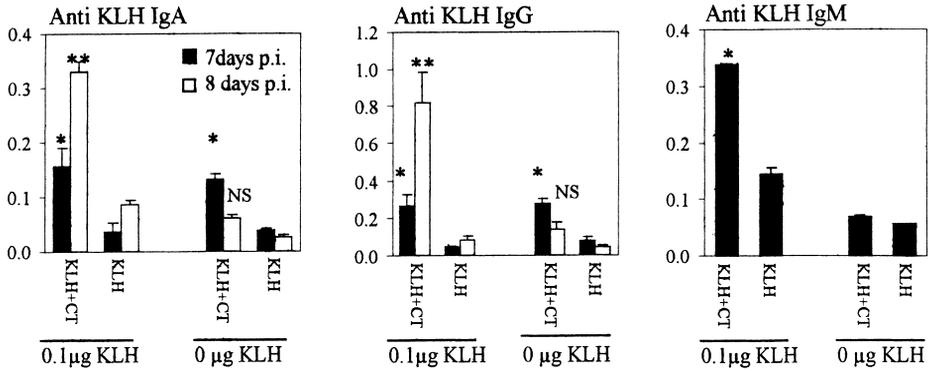


Fig. 3. Adjuvant effect of CT given i.v. on secondary anti-KLH IgG, IgA and IgM production in vitro by spleen cells from chickens after i.v. immunization with KLH seven and eight days p.i. Spleen cells were obtained on days 7 and 8 after i.v. immunization with KLH alone (200 µg) or KLH and CT (4 µg). Cells were cultured in vitro with or without KLH (0.1 µg/ml). Anti-KLH IgG, IgA and IgM were measured in culture supernatant by ELISA. Results are expressed as mean O.D value (\pm SEM) of four chickens (seven days p.i) and eight chickens (eight days p.i). (KLH + TC) values are compared with (KLH) values and statistical significance is indicated (N.S., non significant; * $p < 0.05$; ** $p < 0.01$).

evidence of a selective effect on T-helper subsets by favoring a th2 type of response can be observed (Hornquist and Lycke, 1993). This capacity of CT to prime antigen-specific CD4+ T lymphocytes might be improved by indirect effects, such as the blocking or depletion of suppressive CD8+ T lymphocytes. Indeed, using CD8-deficient mice, Hornquist et al. (1996) proved that the adjuvant action of CT does not require CD8+ T-cells, but is far better in CD8+ cell-depleted mice when given orally and to a lesser extent, when given intravenously. A selective loss of CD8+ cells in mice has been observed in the spleen following intravenous injection of CT (Hornquist and Lycke, 1993) and in the splenic and intestinal epithelial compartments after oral immunization with CT (Elson et al., 1995). Potentiation of antigen-presenting cell function might also be extensively involved in the enhancing effect of CT on immune antibody response. CT has been reported to enhance antigen presentation in vitro in mice, through stimulation of cytokines such as Il-1 and Il-6 and/or enhancement of cell surface molecules such as MHC-II or B7-2, on macrophages (Bromander et al., 1991; Cong et al., 1997), resting B-cells (Papadimitriou et al., 1997) and epithelial cells (Bromander et al., 1993).

In conclusion, CT was proved by our results to exert a strong adjuvant activity in chickens after intravenous administration, leading to a greater and quicker antibody response against the co-administered protein antigen, probably through potentiation of antigen-presenting cell function, as in vitro findings also appeared to suggest (data not shown). Nevertheless, there was no convincing evidence of the capacity of CT to induce IgA switching in chickens, either in vivo (although a peak of antigen-specific IgA did occur), or in vitro (data not shown). Further studies are needed to solve this point. Finally, the hypothesis that the defect in the adjuvant mucosal activity of CT in chickens might be inherent to the characteristics of the bird gut-associated lymphoid tissue remains to be elucidated.

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